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—Review—
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Current Activities of CARD as an International Core Center for Mouse Resources

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Abstract: The Center for Animal Resources and Development (CARD), Institute of Resource Development and Analysis, Kumamoto University was established in 1998 based on recommendations published in the report "Preservation, Supply and Development of Genetically Engineered Animals" by the Ministry of Education, Culture, Sports, Science and Technology. We provide a comprehensive and integrated set of research services designed for the mouse-based biological research community. All services are conducted in accordance with the highest standards of animal health and genetic quality and are delivered to meet researcher's research goals. To promote biological sciences worldwide, we produce genetically engineered mice and exchangeable gene trap ES clones, cryopreserve mouse embryos and sperm, supply these resources, organize training courses to educate people, and form a hub of the domestic and international networks of both mutagenesis and resource centers. Up to now, we have produced more than 600 genetically engineered mouse strains and have more than 1,100 strains and stocks of mice for supply to the scientific community. More than 150 studies using genetically engineered mice produced or supplied by CARD have been published so far. As a founding member of the Federation of International Mouse Resources, the Asian Mouse Mutagenesis and Resource Association, and the International Gene Trap Consortium, we are contributing to the promotion of biological sciences in the world.

Key words: cryopreservation, knockout, mouse, resource, transgenic

Introduction

The Center for Animal Resources and Development (CARD), Kumamoto University was established in 1998 based on recommendations published in the report "Preservation, Supply and Development of Genetically Engineered Mice" by the Subdivision of Resource Study, Council for Science and Technology, Ministry of Education, Culture and Science on July 10, 1997. That report recommended the establishment of at least two centers which could preserve, supply, and develop genetically engineered mice in Japan. At the Institute of Molecular Embryology and Genetics, Kumamoto University, we established the Laboratory of Transgenic Technology in 1992 and started to produce transgenic mice in response to requests from scientists. This was the first trial for

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such a service in Japan. The first transgenic mice were produced by Gordon *et al.* in 1980 [4]. Since then, transgenic mice have been used in many biological studies and have been shown to be a rich resource for such studies. In 1989, the first knockout mice were produced using a homologous recombination technique in embryonic stem (ES) cells [8]. With this procedure we aimed to promote scientific activities through the production of genetically engineered mice. In the mid 1990s, we realized that one transgenic mouse or knockout mouse strain can be used in many different areas of biological science, and that there was a strong demand for the preservation and supply of genetically engineered mouse strains. At the same time, the technology for the cryopreservation of mouse embryos had developed enough to be able to respond to such demand. In 2000, a new facility for embryo cryopreservation was completed and we started to cryopreserve, supply, and develop genetically engineered mice.

Goals of CARD

Our goals are as follows: 1) the establishment of mouse resources unique in Asia by integration of advanced technologies in production, cryopreservation, and the supply of genetically engineered mice; 2) the development of technologies relevant to these areas to keep our techniques advanced; 3) the establishment of a supply system between our center and other institutions for cryopreserved embryos or gametes to protect against infection which may arise through the transfer of live mice; 4) the training and education of workers and researchers in this field, especially those involved in reproductive engineering; and 5) the formation of domestic and international networks to promote biological sciences globally. To accomplish these goals, we became a founding member of the Federation of International Mouse Resources (FIMRe) [3]. FIMRe is a collaborating group of Mouse Repository and Resource Centers worldwide whose collective goal is to archive and provide strains of mice as cryopreserved embryos and gametes, ES cell lines, and live breeding stock to the research community. Goals of the FIMRe are (1) Coordinate repositories and resource centers to archive valuable genetically defined mice and ES cell lines being created worldwide and meet research demand for these genetically defined mice and ES cell lines, (2) Establish consistent animal health standards of the highest quality in all resource centers, (3) Provide genetic verification and quality control for genetic background and mutations, (4) Provide resource training to enhance user ability in utilizing cryopreserved resources.

In addition, we organized the Asian Mouse Mutagenesis and Resource Association (AMMRA) in 2006. The AMMRA is a collaborative group of Mouse Mutagenesis and Resource Centers in Asia. Its mission is "To promote mouse mutagenesis projects and to facilitate access to mouse resources in Asia". Its goals are "The use of mouse models for understanding genome function and the improvement of human health". The founding members of AMMRA are (1) Biological Resource Center, Singapore, (2) National Laboratory Animal Center, Taipei, (3) National Resource Center for Mutant Mice, Nanjing University, Nanjing, (4) Shanghai Institute of Biological Sciences, Shanghai, (5) Nanfang Center for Model Organisms, Shanghai, (6) Peking University-BLARC, Beijing, (7) Beijing Institute of Laboratory Animal Science, CAMS, PUC, Beijing, (8) Bio-Evaluation Center, KRIBB, Daejeon, (9) Riken BioResource Center, Tsukuba, (10) Center for Animal Resources and Development, Kumamoto.

Characteristics of the Mouse

Development of the laboratory mouse as a research model really began with genetic experiments in the early 1900s. Today, a large number of inbred strains of mice or various strains of mice with spontaneous or induced mutations (i.e., transgenics, targeted mutations, chemically induced mutations) have been produced in a variety of laboratories worldwide. They are prized for many qualities, including their small size, short generation time, and ease of breeding within the laboratory. The fact that they are genetically the best characterized of all mammals increases their value for all fields of study.

Technical Services Available at CARD

According to our aims, we provide a comprehensive,

Table 1. Production, cryopreservation, supply, and cleaning of genetically engineered mice at CARD since 1998

	Tg	Chimera	Total	No. of strains cryopreserved	No. of strains supplied	No. of strains cleaned
1998	67	6	73			3
1999	22	1	23			53
2000	66	11	77	144	4	62
2001	60	15	75	97	10	73
2002	59	21	80	67	34	47
2003	61	23	84	89	33	20
2004	44	14	58	116	58	65
2005	43	18	61	111	39	83
2006	23	13	36	153	42	83
2007	25	7	32	207	35	48
2008	37	6	43	142	53	106
Total	507	135	642	1,126	308	643

integrated, and highly customizable set of research services designed for the mouse-based biomedical research community. All services are conducted in accordance with the highest standards of animal health and genetic quality and are delivered to meet user's schedule and research goals. The intellectual property right of each resource belongs to the depositor, not to our center. Users should consider this issue before submitting a request for resources, and undertake signed agreements to any conditions attached to a resource.

(1) Production of genetically engineered mice

Upon requests by researchers, we produce either transgenic mice or chimeric mice. So far, we have produced 507 transgenic mouse lines and 135 chimeric mouse lines (Table 1). Researchers should prepare and send DNA constructs for microinjection into fertilized eggs. We usually use fertilized eggs obtained from C57BL/6 mice. We microinject DNA into fertilized mouse eggs and transfer them into oviducts of foster mothers. We rear new born mice up to 4 weeks of age and then send these mice to requesters. So, researchers need to carry out screening for transgenic mice at their facilities. For chimeric mouse production, researchers should prepare knockout ES clones at their facilities. We make chimeric mice by injecting targeted ES cells into blastocysts, which are then transferred to the uteri of foster mothers. We send all chimeric mice to requesters at 4 weeks of age. It should be noted that researchers should have permission for recombinant DNA experiments at their institutions.

(2) Cryopreservation of embryos and sperm

For deposit, researchers should send their mice to CARD. The numbers of mice that should be shipped to CARD for embryo freezing is as follows. For genetically engineered mouse strains, researchers should send at least 3 homozygous or 3 heterozygous male mice. Alternatively, researchers can send at least 5 homozygous males and 20 homozygous females if they desire cryopreservation of homozygous embryos. For other mouse strains, we need at least 5 males and 20 females for cryopreservation.

We generally produce embryos from these mouse strains using *in vitro* fertilization. Ten straws containing spermatozoa and 8 tubes containing 40 two-cell embryos are cryopreserved for each strain. We use the simple vitrification method for embryo freezing [6] and the Nakagata method for sperm freezing [5]. For quality control, some of the frozen embryos are thawed to check if the frozen embryos develop into viable young and whether or not the developed mice are microbiologically clean. For genetically engineered mouse strains, transgene transmission is confirmed by PCR analysis of genomic DNA from tail tissue of the produced mice.

Currently our center has 1,126 strains and stocks of mice. These include inbred mouse strains, spontaneous

mutants, transgenic mice, and knockout mice. CARD R-BASE (Resource Database) provides resource-related strain, gene and reference information (http://cardb.cc.kumamoto-u.ac.jp/transgenic/index.jsp).

(3) Supply of mouse strains and frozen embryos/sperm

Cryopreserved embryos or recovered populations from cryopreserved embryos are supplied to the scientific community. The cost is141,750 JY or 109,200 JY for supply of recovered mice or frozen embryos, respectively. The application procedure for the supply of laboratory mice is shown on our web page (http://card.medic.kumamoto-u.ac.jp/card/english/sigen/gyoumu/ebank/index.htm). We have already supplied 90 strains of cryopreserved embryos and 218 strains of mice worldwide.

(4) Exchangeable gene trap clones (EGTC)

Our center has more than 600 exchangeable gene trap ES clones. In general, gene trapping is a high-throughput approach that is used to introduce insertional mutations across the genome in mouse embryonic stem (ES) cells. In addition to generating standard loss-of-function alleles, newer gene trap vectors offer a variety of post-insertional modification strategies for the generation of other experimental alleles. We are using the exchangeable gene trap method for isolation of gene trap clones [1, 2, 9]. This method has a great advantage. The exchangeable gene trap vector is usually inserted around the exon containing the ATG codon in a mouse endogenous gene, resulting in the null mutation. As the β-geo gene is flanked by lox71 and loxP, the β-geo gene can be replaced with the gene of interest by electroporating a new vector containing the gene of interest flanked by lox66 and loxP together with a Cre expression vector. The newly introduced gene of interest can be expected to be expressed under the control of the trapped gene. Using this system, a mouse gene can be replaced with a homologous human gene, leading to the production of a humanized mouse at the gene level. These clones can be accessed through the EGTC database (http://egtc.jp/view/index). As we are also the founding member of the international gene trap consortium (IGTC) [7], the EGTC database is connected to the integrated database of the IGTC. The IGTC database contains all publicly available gene trap cell lines, which are available on a non-collaborative basis for nominal handling fees. Researchers can search and browse the IGTC database for cell lines of interest using accession numbers or IDs, keywords, sequence data, tissue expression profiles, and biological pathways. At the moment, the IGTC database has 380,863 cell lines. IGTC members are BayGenomics (USA), Centre for Modelling Human Disease (Toronto, Canada), Embryonic Stem Cell Database (University of Manitoba, Canada), German Gene Trap Consortium (Germany), Sanger Institute Gene Trap Resource (Cambridge, UK), Soriano Lab Gene Trap Database (Mount Sinai School of Medicine, New York, USA), Texas Institute for Genomic Medicine—TIGM (USA), TIGEM-IRBM Gene Trap (Naples, Italy), and our institution, Exchangeable Gene Trap Clones (Kumamoto University, Japan).

(5) Other resources

In the Gene Technology Center, which also belongs to the Institute of Resource Development and Analysis, we started the GTC P-stock service in April 2004. This is not a plasmid bank; however, we do store valuable transgenes or targeting constructs for homologous recombination and supply them upon request (http://gtc.egtc.jp/view/pstock/index).

(6) Courses and education

CARD sponsors stimulating courses and conferences on our campus at Honjo in Kumamoto and at other locations inside and outside Japan. CARD training courses for reproductive engineering techniques were introduced in November 2000. Spring and autumn courses are staged each year at CARD. The courses teach the following techniques:1) Preparing and Assembling Pipettes for Embryo Handling, 2) *In Vitro* Fertilization, 3) Simple Vitrification of Mouse Embryos, 4) Vitrification and Transplantation of Mouse Ovaries, 5) Cryopreservation of Mouse Spermatozoa, 6) *In Vitro* Fertilization using Cryopreserved Spermatozoa, 7) Collecting Two-Cell-Stage Embryos, 8) Vasectomy for the Creation of Sterile Males, 9) Embryo Transfer into the Oviduct, 10) Production of Chimeric Mice by 8-Cell Aggregation, 11) Embryo Transfer into the Uterus, and 12) Caesarean Section and Fostering. We sometimes hold the training course at other venues inside and outside Japan.

Popular Strains: Characteristic Features and Their Use for Research

Table 2 shows the mouse strains which are requested by many researchers. A recent trend is that requests for Cre mice are increasing substantially due to the performance of its conditional knockout.

Among Cre mice, P0-Cre is one of the most frequently requested strains. Although this strain of mouse has been described in a published manuscript [10], we would like to briefly introduce the characteristics of this mouse. Neural crest cells are embryonic, multipotent stem cells that give rise to various cell/tissue types and thus serve as a good model system for the study of cell specification and mechanisms of cell differentiation. For analysis of neural crest cell lineage, transgenic mice harboring a Cre gene driven by a promoter of protein 0 (P0) were generated. To detect and visualize the Cre-mediated DNA recombination in neural crest cells and derivatives, we utilized another transgenic (Tg) line with the CAG-CAT-Z indicator construct [10]. This Tg line carries a *lacZ* reporter gene downstream of a chicken β-actin promoter and a "stuffer" fragment flanked by two *loxP* sequences. The *lacZ* is expressed only when the stuffer is removed by the action of Cre recombinase. Using these transgenic mice, we demonstrated that a subset of migrating neural crest cells and a wide variety of cells in the neural crest cell lineage could be marked by *lacZ* expression. Thus, the use of this system may facilitate many interesting experiments, including lineage analysis, purification, and manipulation of the mammalian neural crest cells. Also, this cell-type-specific transgenesis system should facilitate functional analysis of genes of interest in the neural crest cell lineage. Up to now, this strain of mouse has been used in many studies and is now one of the most useful Cre-driver mice.

Deposition and Request for Mouse Strains and EGTC

An outline of the deposition and request procedures for mouse strains is illustrated in Fig. 1. Information on the application procedure is available on the web page (Fig. 2) (http://card.medic.kumamoto-u.ac.jp/card/english/index.html). As we are one of the founding members of FIMRe (http://www.fimre.org/), we also offer our mouse data to the integrated database IMSR (International Mouse Strain Resources) (http://www.informatics.jax.org/imsr/index.jsp). Thus, researchers also can find mouse strains using these databases.

Table 2. Frequently requested mouse strains

ID: 148	C57BL/6J-Tg(PO-Cre)94Imeg
ID: 250	Tg(K5-Cre)
ID: 709	B6;C3H-Tg(K19-Wnt1/K19-Ptgs2/K19-Ptges)
ID: 428	C57BL/6N-Tg(CAG-AURKA(WT)Card
ID: 196	B6;129-Synd4^{tm1}
ID: 189	Tg(E/nestin:EGFP-50)
ID: 175	Tg(E/nestin:EGFP-25)
ID: 91	Tg(CAG-Cre)
ID: 89	Tg(lck-Cre)
ID: 509	B6;D2-Tg(CAG-CAT-EGFP)39Miya
ID: 355	C57BL/6-CD9^{tm1};Tg(ZP3-EGFPCD9)
ID: 312	B6;CB-Dtr$^{tm2(lox)}$
ID: 290	C57BL/6 -Tg(Act-EGFP)C14-Y01-FM131Osb
ID: 88	C57BL/6-Rag1$^{tm1(GFP)Imku}$

Major Achievement: Publication

More than 150 studies using genetically engineered mice produced by CARD, have been published so far. Among these some representative manuscripts are listed in Table 3. This list clearly suggests that technical services in mouse embryo manipulation and reproductive engineering contribute greatly to the promotion of science.

Related Information

JMSR (http://www.shigen.nig.ac.jp/mouse/jmsr/top.jsp): The Japan Mouse/Rat Strain Resources Database (JMSR) is a searchable online database of mouse/rat strains and stocks available in Japan. The JMSR was developed in 2001 through discussions with the Mouse Genetic Resources Subcommittee (http://www.shigen.nig.ac.jp/shigen/grc/grc_mouse.jsp). The objective of this database is to provide a portal site which will help users to locate and obtain mouse/rat resources. This site is maintained by the Genetic Informatics Laboratory, National Institute of Genetics in close cooperation with data providers.

IGTC (http://www.genetrap.org/): The International

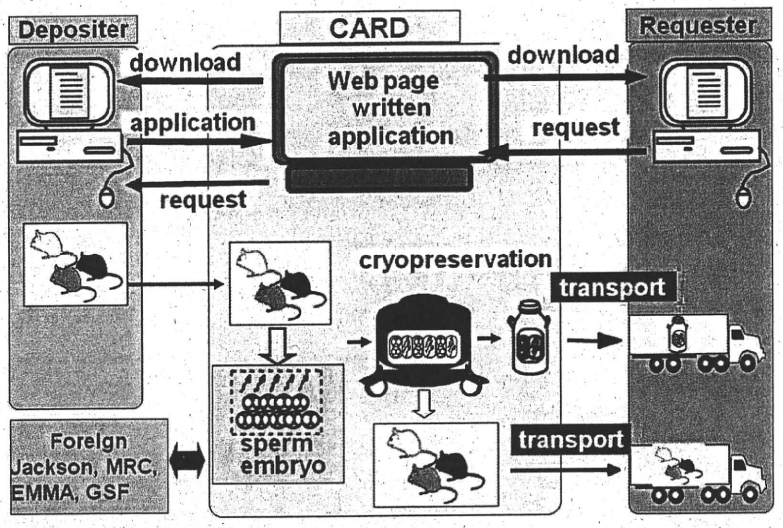

Fig. 1. Outline of deposition and request procedures for mouse strains. Information on the application procedure and necessary documents are available on the web page. Mouse embryos or sperm obtained from mice sent from depositors are cryopreserved and stored in liquid nitrogen tanks. Frozen embryos or live mice obtained by transfer of thawed embryos into foster mothers can be sent to requesters.

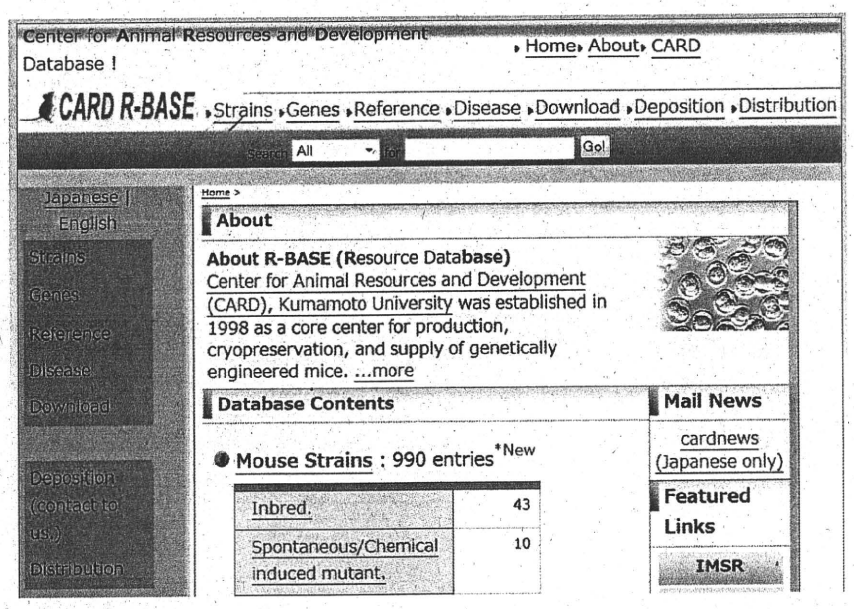

Fig. 2. Web page of CARD R-BASE. Only part of the web page is shown in this figure. Both Japanese and English versions are available.

Gene Trap Consortium (IGTC) consists of laboratories around the world working together to generate a public library of mutated murine ES cell lines. Such cell lines can be obtained on a non-collaborative basis by scientists interested in generating reporter-tagged, loss-of-function mutations in mice. In addition to loss of function, new-

Table 3. Selected publications

Ohnishi, N. *et al. Proc. Natl. Acad. Sci. U.S.A.* 105: 1003–1008, 2008.
Sato, T. *et al. Nature* 448: 366–369, 2007.
Imai, T. *et al. Science* 314: 657–661, 2006.
Serizawa, S. *et al. Cell* 127: 1057–1069, 2006.
Shim, J.H. *et al. Genes Dev.* 19: 2668–2681, 2005.
Terada, K. *et al. EMBO J.* 24: 611–622, 2005.
Tachibana, M. *et al. Genes Dev.* 19: 815–826, 2005.
Ohmuraya, M. *et al. Gastroenterology* 129: 696–705, 2005.
Itoh, H. *et al. Gastroenterology* 127: 1423–1435, 2004.
Yamazaki, S. *et al. J. Cell Biol.* 163: 469–475, 2003.
Ishida, D. *et al. Cancer Cell* 4: 55–65, 2003.
Serizawa, S. *et al. Science* 302: 2088–2094, 2003.
Wakabayashi, Y. *et al. Nat. Immunol.* 4: 533–539, 2003.
Yokosuka, T. *et al. J. Exp. Med.* 195: 991–1001, 2002.
Pepys, M.B. *et al. Nature* 417: 254–259, 2002.
Tachibana, M. *et al. Genes Dev.* 16: 1779–1791, 2002.
Yamauchi, T. *et al. Nat. Genet.* 30: 221–226, 2002.
Hasegawa, S. *et al. Proc. Natl. Acad. Sci. U.S.A.* 99: 297–302, 2002.
Yamaguchi, S. *et al. Nature* 409: 684, 2001.
Nishimura, H. *et al. J. Exp. Med.* 191: 157–169, 2000.
Hisahara, S. *et al. EMBO J.* 19: 341–348, 2000.
Nakai, A. *et al. EMBO J.* 19: 1545–1554, 2000.
McIlroy, D. *et al. Gene. Dev.* 14: 549–558, 2000.
Serizawa, S. *et al. Nat. Neurosci.* 3: 687–692, 2000.
Ishiguro, K. *et al. J. Clin. Invest.* 106: 873–878, 2000.
Shinkura, R. *et al. Nat. Genet.* 22: 74–77, 1999.
Terauchi, Y. *et al. Nat. Genet.* 21: 230–235, 1999.
Watanabe, N. *et al. J. Exp. Med.* 190: 461–469, 1999.
Ihara, Y. *et al. Proc. Natl. Acad. Sci. U.S.A.* 95: 2526–2530, 1998.
Fujii, H. *et al. EMBO J.* 17: 6551–6557, 1998
Watanabe, D. *et al. Cell* 95: 17–27, 1998.
Sawada, S. *et al. J. Exp. Med.* 187: 1439–1449, 1998.

er gene trap vectors offer a variety of post-insertional modification strategies to allow for the generation of other experimental alleles. The cooperative goal of the IGTC is to generate an international resource representing all or most genes in the mouse genome, and to provide the bioinformatics and logistical support to make the resource valuable and available to scientists.

IMSR (http://www.informatics.jax.org/imsr/index.jsp): The International Mouse Strain Resources (IMSR) is a searchable online database of mouse strains and stocks available worldwide, including inbred, mutant, and genetically engineered mice. The goal of the IMSR is to assist the international scientific community in locating and obtaining mouse resources for research. The data content found in the IMSR is as it was supplied by data provider sites.

EUCOMM (http://www.eucomm.org/): The European Conditional Mouse Mutagenesis Integrated Project (EUCOMM) is funded by the European Union Framework 6 programme. The goal of EUCOMM is to generate a collection of up to 13,000 mutated genes in mouse C57BL/6N embryonic stem (ES) cells using conditional gene trapping and gene targeting approaches. This library will enable mouse mutants to be established worldwide in a standardized and cost-effective manner, making mouse mutants available to a much wider biomedical research community than has been possible previously.

NorCOMM (http://norcomm.phenogenomics.ca/): NorCOMM (North American Conditional Mouse Mutagenesis project) is a large-scale research initiative focused on developing and distributing a library of mouse embryonic stem (ES) cell lines carrying single conditional knockout mutations across the mouse genome.

KOMP (http://www.nih.gov/science/models/mouse/knockout/): The Knockout Mouse Project is a trans-NIH initiative that aims to generate a comprehensive and public resource comprised of mouse embryonic stem (ES) cells containing a null mutation in every gene in the mouse genome.

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Relationship of strain-dependent susceptibility to experimentally induced acute pancreatitis with regulation of Prss1 and Spink3 expression

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To analyze susceptibility to acute pancreatitis, five mouse strains including Japanese Fancy Mouse 1 (JF1), C57BL/6J, BALB/c, CBA/J, and C3H/HeJ were treated with either a cholecystokinin analog, cerulein, or a choline-deficient, ethionine-supplemented (CDE) diet. The severity of acute pancreatitis induced by cerulein was highest in C3H/HeJ and CBA/J, moderate in BALB/c, and mildest in C57BL/6J and JF1. Basal protein expression levels of the serine protease inhibitor, Kazal type 3 (Spink3) were higher in JF1 and C57BL/6J mice than those of the other three strains under normal feeding conditions. After treatment with cerulein, expression level of Spink3 increased remarkably in JF1 and mildly in C57BL/6J, BALB/c, CBA/J, and C3H/HeJ strains. Increased proteinase, serine, 1 (Prss1) protein expression accompanied by increased trypsin activity with cerulein treatment was observed in susceptible strains such as CBA/J and C3H/HeJ. Similar results were obtained with a CDE diet. In the 3 kb Spink3 promoter region, 92 or 8 nucleotide changes were found in JF1 or C3H *vs* C57BL/6J, respectively, whereas in the Prss1 promoter region 39 or 46 nucleotide changes were found in JF1 or C3H *vs* C57BL/6J, respectively. These results suggest that regulation of Prss1 and Spink3 expression is involved in the susceptibility to experimentally induced pancreatitis. The JF1 strain, which is derived from the Japanese wild mouse, will be useful to examine new mechanisms that may not be found in other laboratory mouse strains.
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KEYWORDS: serine protease inhibitor, Kazal type 3 (Spink3); proteinase, serine, 1 (Prss1); single-nucleotide polymorphisms (SNPs)

Over 450 inbred strains of mice have been described,[1] providing a wealth of different genotypes and phenotypes for studying human diseases. Actually, the use of various breeding strategies in combination with positional cloning and positional candidate gene approach has led to the discovery of many genes that underlie human disease.[2] The Japanese wild mouse, belonging to *Mus musculus molossinus*, has several genetic characteristics clearly distinguishable from the European wild mouse, derived from *M.m. domesticus*. These subspecies were separated about one million years ago and about 1% of their genome sequences are different.[3–8] Therefore, strains MSM/Ms[9] and Japanese Fancy Mouse 1 (JF1),[10] which were established from *M.m. molossinus*, are powerful genetic resources to analyze disease processes.

Many inbred strains have been bred for specific phenotypes. C57BL/6J mice are susceptible to high-fat diet-induced type II diabetes.[11] JF1 mice are especially sensitive to high-fat diet-induced diabetes and obesity, whereas MSM/Ms mice are resistant.[12] To date, however, there is little information about the difference in severity of pancreatitis among inbred strains of mice.

Acute pancreatitis is an important disease that can be triggered by a variety of factors, including excessive alcohol consumption,[13–16] obstruction of the ampulla of Vater by gall stones,[17,18] and genetic factors.[19,20] Hereditary chronic pancreatitis is a rare form of early onset chronic pancreatitis, characterized by the onset of recurrent attacks of acute pancreatitis in childhood, and frequently progresses to chronic pancreatitis. One type of the hereditary pancreatitis is caused by a mutation in the proteinase, serine, 1 (PRSS1) gene, encoding cationic trypsinogen. PRSS1 mutation results in an amino-acid substitution in the autolytic domain of

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trypsin. Thus, the mutation blocks autolysis and results in continuous trypsin activity.[21,22] In addition, mutation of the trypsin-specific inhibitor, serine protease inhibitor, Kazal type 1 (SPINK1), has been found to be associated with chronic pancreatitis as well.[23] We earlier showed that Spink3 (mouse homolog of human SPINK1) has dual functions for trypsin inhibition: one as a trypsin inhibitor by direct binding to trypsin[24] and another as a suppressor of autophagy, which is involved in trypsinogen activation.[25,26] In summary, mutations in PRSS1[27] and SPINK1[19] genes are acknowledged as genetic risk factors for pancreatitis in human patients.

To address whether genetic background can affect the development of acute pancreatitis in relation to expression of Prss1 and Spink3, the susceptibility of acute pancreatitis was compared among five inbred mouse strains including JF1 in two models of experimental acute pancreatitis, cerulein-induced and choline-deficient ethionine-supplemented (CDE) diet-induced acute pancreatitis. We found that there were significant differences in susceptibility to acute pancreatitis among mouse strains, and that susceptibility to acute pancreatitis was negatively or positively related with expression levels of Spink3 or Prss1, respectively. In addition, sequence differences in Spink3 promoter regions between JF1 and other strains were considered to be involved in differences in expression levels.

MATERIALS AND METHODS

Mouse Strains

All procedures were approved by the Animal Care and Use Committee of Kumamoto University. The five mouse strains were used in the following experiments: JF1 (Riken BioResource Center, Tsukuba, Japan), C57BL/6J, CBA/J, and BALB/c (CLEA Japan, Inc. Tokyo, Japan), and C3H/HeJ (Charles River Laboratories Japan, Inc. Yokohama, Japan). For every experiment, five mice in each strain were assigned to either a control or experimental group.

Cerulein-Induced Pancreatitis

After an overnight fast, mice were given hourly intraperitoneal injections of either saline (control) or cerulein (50 μg/kg) (Sigma-Aldrich Corp, Tokyo, Japan) for 12 h. One hour after the last injection, serum and the pancreas were isolated for the following studies.

CDE Diet-Induced Pancreatitis

Composition of CDE chow was described earlier.[28] Mice were fasted for 24 h and then fed with either CDE or control (regular laboratory chow) diet for 72 h. Then, animals were fed with regular laboratory chow for 12 h before they were killed.

Histological Examination and Pathologic Scoring

For histological analysis, pancreatic tissue was fixed overnight in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The extent of injury was estimated using a method described earlier with some minor modifications.[29] Briefly, 10 randomly chosen microscopic fields were examined for each pancreas specimen, and the total surface of the slide was scored for five different variables determining severity of inflammation: edema (E), hemorrhage (H), inflammatory cell infiltration (I), acinar cell vacuolization (V), and acinar cell necrosis (N). Total scores for these five parameters were obtained in each group after mapping the pancreas into 10 fields and evaluating each field independently.

Serum Amylase Activity

Mouse blood was used to measure pancreatic amylase activity using substrate, 2-chloro-4-nitrophenyl-4-galactopyranosylmaltoside (Gal-G2-CNP) (CicaLiquid-N p-AMY, Kanto Chemical Co., Inc. Tokyo, Japan).

Measurement of Trypsin Activity

Measurement of trypsin activity was performed as described earlier.[25]

Northern Blot Analysis

Total RNAs were extracted from the pancreas with Sepasol (Nacalai Tesque, Kyoto, Japan). For making digoxigenin-labeled RNA probes (Roche Molecular Biochemicals, Germany), mouse Spink3 and Prss1 probes were derived from mouse pancreas RNA by reverse transcriptase PCR using the following nucleotide sequences: mPsti1 (agttcttctggcttttgcaccc) and mPsti26 (cccacgttgcctttcattacgg); Prss1: mPrss1-s1 (taaaggcaggcttccatccagg) and mPrss1-a1 (tgacagtgactgcagagggatt). cDNA was subsequently subcloned into a pGEM-T easy vector (Promega, Madison, WI, USA).

Sequence Analysis

Both cDNAs and the promoter regions of Spink3 and Prss1 gene were amplified by PCR using TaKaRa LA Taq polymerase mix (Takara Bio Inc., Kyoto, Japan). Primers used in the RT–PCR included the following sequences: mPsti1 (agttcttctggcttttgcaccc) and mPsti26 (cccacgttgcctttcattacgg) for Spink3; mPrss1-s1 (taaaggcaggcttccatccagg) and mPrss1-a1 (tgacagtgactgcagagggatt) for Prss1. Three pairs of primers were applied to sequence the 3 kb promoter region of each gene. Sequencing was performed using the Big Dye Terminator Cycle Sequencing ready kit and an ABI 310 Genetic Analyzer (Applied Biosystems).

Western Blot Analysis

Western blot analysis was carried out according to the method described earlier.[25] Primary antibodies to the following antigens were used at the indicated dilutions: Spink3 (Transgenic Inc., Kumamoto, Japan), 1:1000; Prss1 (Nordic immunological laboratories, Tilburg, Netherlands), 1:1000; amylase (Santa Cruz Biotechnology, CA, USA), 1:1000; and light chain 3 (LC3) (MBL International, Woburn, MA, USA),

1:1000. An anti-rabbit secondary immunoglobulin G antibody conjugated with horseradish peroxidase (Amersham Biosciences Corp, Piscataway, NJ, USA) was used to detect all proteins. Intensities of the bands were quantified by densitometry using ImageJ software (version 1.38, a program inspired by NIH image; http://rsb.info.nih.gov/ij/docs/index.html).

Statistical Analysis

All values are presented as mean ± s.d. (range, 95% CI), and statistical analysis was performed applying unpaired Student's *t*-tests. $P<0.05$ was considered to be a statistically significant difference.

RESULTS

Cerulein-Induced Pancreatitis

The pancreas of all strains of mice injected with normal saline showed no histological changes, whereas cerulein treatment induced variable degrees of acute pancreatitis among these five strains (Figure 1). C57BL/6J mice showed the mildest pancreatitis (Figure 1g and h), whereas C3H/HeJ mice displayed the most severe pancreatitis (Figure 1s and t). BALB/c and CBA/J mice showed a moderate degree of pancreatitis (Figure 1k, l, o and p). Acute pancreatitis in JF1 mice was obviously milder than pancreatitis in BALB/c and CBA/J mice, yet more severe than C57BL/6J with evidence of acinar cell necrosis (Figure 1c). Further calculations of semi-quantitative pathologic scores and statistical analysis strongly supported this statistical difference in susceptibility (Figure 1u and v). Levels of amylase coincide with histological findings, highest in C3H/HeJ, followed by CBA/J and BALB/c, and lowest in C57BL/6J and JF1 with statistically significant differences ($P<0.05$) (Figure 1w). Interestingly, serum levels of amylase in JF1 mice were lowest in spite of the presence of low grade of acinar cell necrosis when compared with C57BL/6J mice.

CDE Diet-Induced Pancreatitis

In CDE diet-induced pancreatitis, the general histological appearance of pancreatic tissues was much milder than those in cerulein-induced pancreatitis. Acinar cells presented almost no obvious histological changes in JF1, C57BL/6J or BALB/c mice (Figure 2c, d, g, h, k and l). C3H/HeJ mice, however, showed signs of obvious necrosis in acinar cells together with infiltration of inflammatory cells and slight vacuolization of acinar cells (Figure 2s and t). A mild degree of acinar cell necrosis was observed in CBA/J mice (Figure 2o and p). Peripancreatic hemorrhage was a frequent sign observed in this model as shown in Figure 2l, whereas fewer inflammatory cells were found within the pancreatic interstitium (Figure 2s). Pathologic scores were quite consistent to the microscopically gross observation (Figure 2u and w). Levels of serum amylase were proportional to these histological findings (Figure 2w).

Basal Expression Levels of Spink3 in Mice under Normal Feeding

Basal expression levels of Spink3 without induction were examined by western blot analysis in each strain of mice. Interestingly, results showed that expression of Spink3 was statistically higher in JF1 and C57BL/6J than the other three strains of mice that were susceptible to pancreatitis (Supplementary Figure 1).

Expression of Spink3 and Prss1 after Treatment with Cerulein

We examined the levels of mRNA and protein expressions of Spink3 and Prss1 without and with cerulein treatment. Without induction, CBA/J mice had lower Spink3 mRNA levels than those in other strains (Figure 3a and b). With cerulein treatment, Spink3 mRNA expression significantly increased in JF1, mildly in C57BL/6J, but decreased in BALB/c and C3H/HeJ strains (Figure 3a and c).

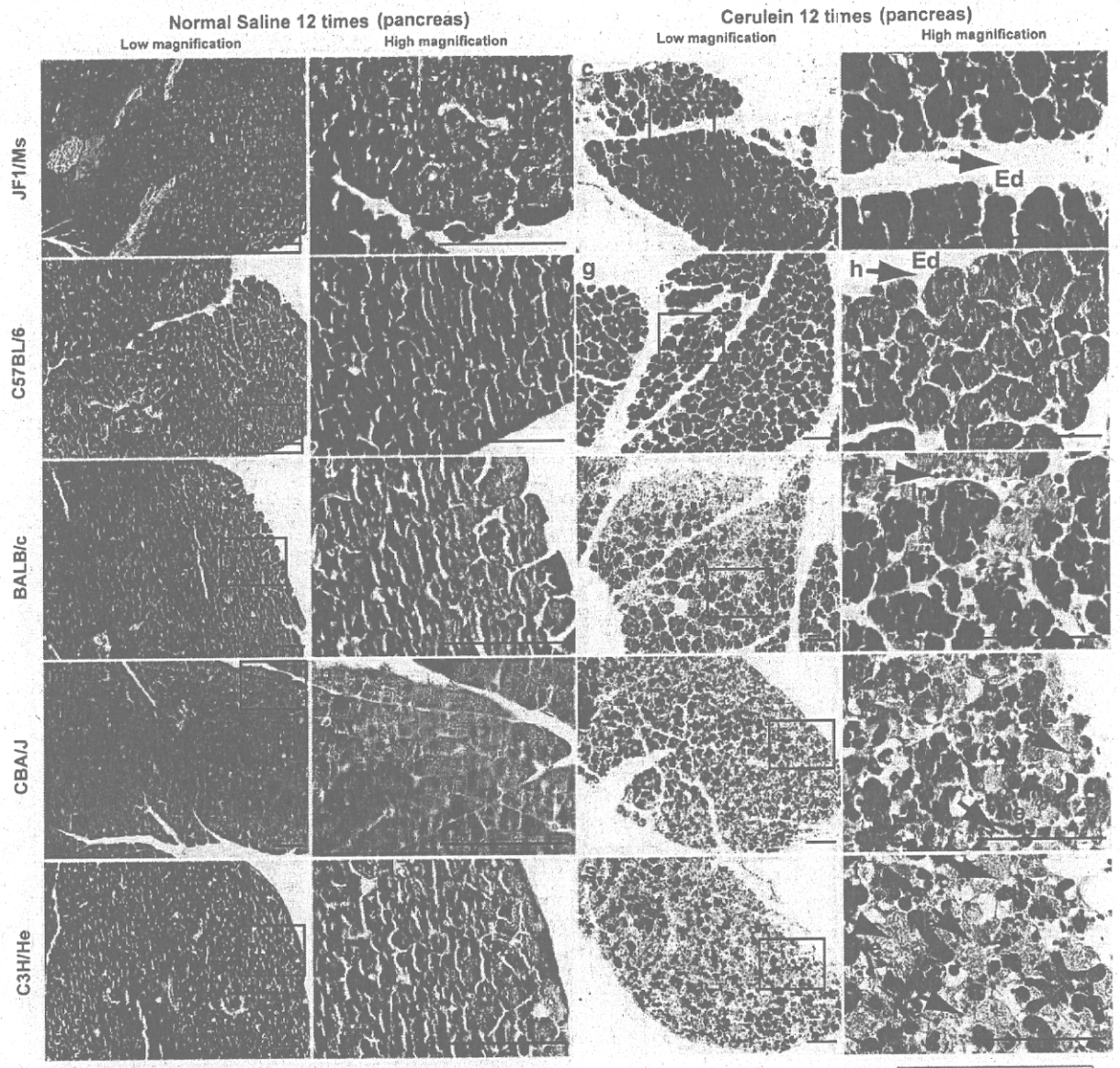
Without cerulein treatment, BALB/c mice had higher Prss1 mRNA levels than those in other strains (Figure 3a and b). After cerulein treatment, Prss1 mRNA levels in JF1, C57BL/6J, and BALB/c remained unchanged, but decreased in CBA/J and C3H/HeJ mice (Figure 3a and c). Expression G3PDH was notably augmented with cerulein treatment (Figure 3a), suggesting that this gene expression could not be used as reference loading control. Thus, we used 18S or 28S rRNA as reference RNAs.

Then, protein expression for Spink3 and Prss1 was examined by western blot analyses. Notably, Spink3 levels in JF1 mice decreased after overnight starvation (Figure 3e) when compared with normal feeding (see Supplementary Figure 1), although levels in other strains were unchanged after starvation. Without treatment, levels of Spink3 in C57BL/6J mice were higher than those in the other four strains (Figure 3d and e). With cerulein treatment, Spink3 expression was increased significantly in JF1, moderately in C57BL/6J and BALB/c, and slightly in CBA/J and C3H/HeJ (Figure 3d and f).

The level of Prss1 protein expression was lower in CBA/J and C3H/HeJ than those in the other three strains without induction (Figure 3d and e). However, levels of Prss1 increased significantly in BALB/c, CBA/J, and C3H/HeJ with cerulein treatment, whereas no notable changes were observed in JF1 and C57BL/6 strains (Figure 3d and f). Taken together, these results suggest that induction levels of Spink3 and Prss1 were negatively and positively related to susceptibility of cerulein-induced pancreatitis, respectively.

Expression of Spink3 and Prss1 with CDE Diet

When given a normal diet, levels of Spink3 mRNA in C57BL/6, CBA/J, and C3H/HeJ mice were higher than those in JF1 and BALB/c (Figure 4a and b). In the CDE diet model, Spink3 mRNA expression was significantly increased in JF1 mice, but mildly increased in C57BL/6J and CBA/J (Figure 4a and c).



u Pathologic Scores in Cerulein-induced acute pancreatitis

M. Strain	Treat	No. of Mice	E	H	I	V	N	Total Scores	Mean ± SD
JF1/Ms	Saline	5	2	0	0	0	0	2	0.2 ± 0.12
	Cerulein	5	5	0	3	1	3	12	2.28 ± 0.49
C57BL/6	Saline	5	0	0	0	0	0	0	0.2 ± 0.12
	Cerulein	5	2	0	0	0	0	2	0.36 ± 0.11
BALB/c	Saline	5	0	0	0	0	0	0	0 ± 0
	Cerulein	5	4	0	4	3	7	18	3.6 ± 0.4
CBA/J	Saline	5	1	0	0	0	0	1	0.2 ± 0.18
	Cerulein	5	5	2	7	3	12	29	5.8 ± 1.02
C3H/He	Saline	5	2	0	0	0	0	2	0.2 ± 0.07
	Cerulein	5	7	1	9	1	20	38	7.6 ± 0.8

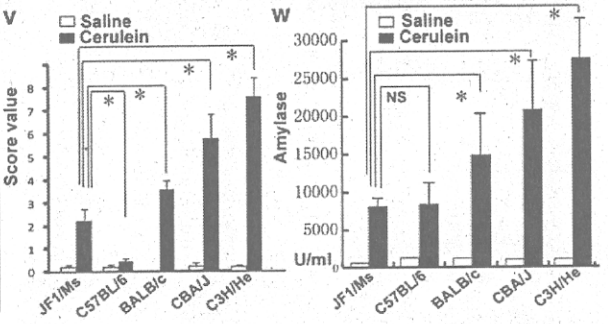
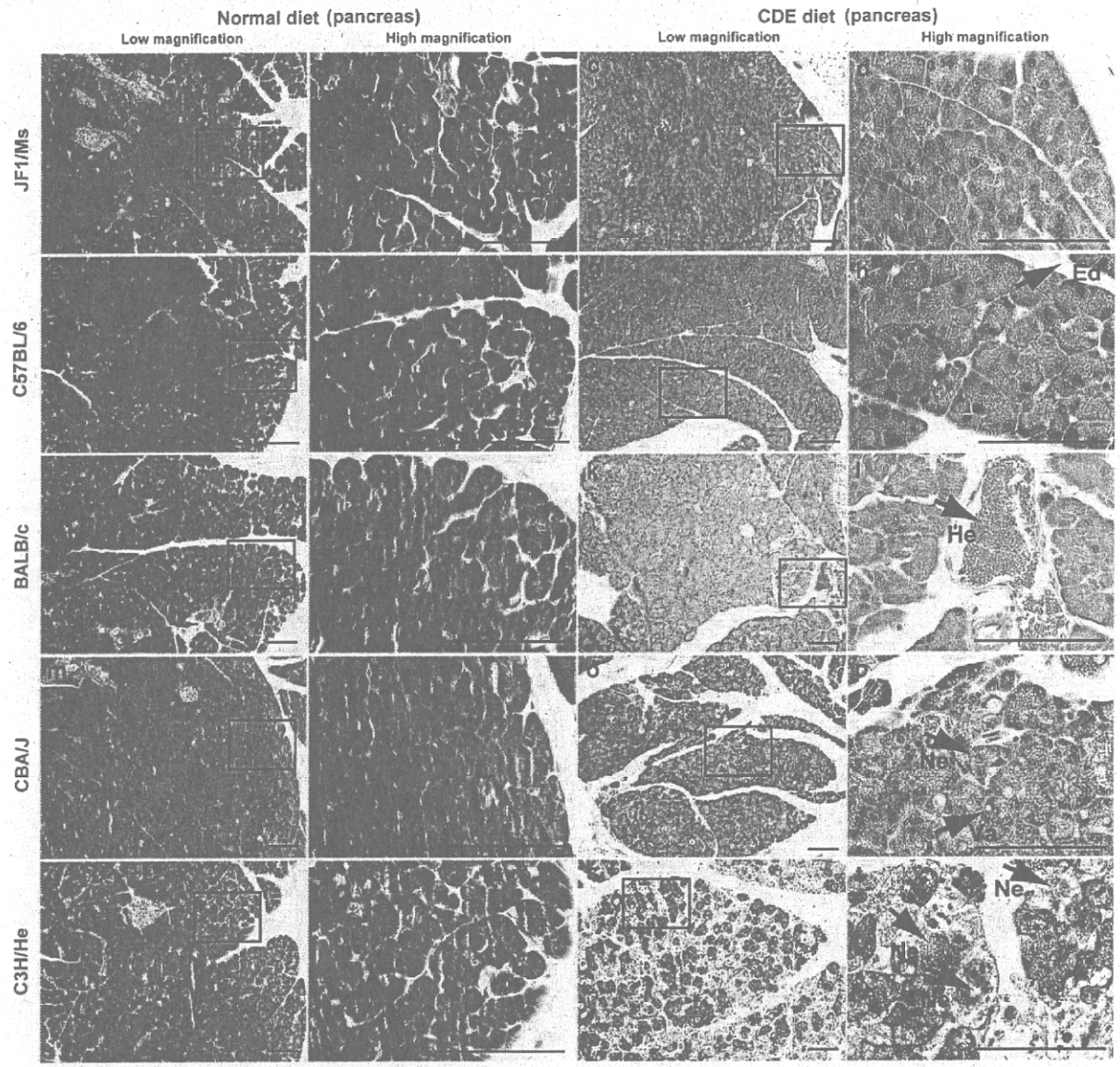
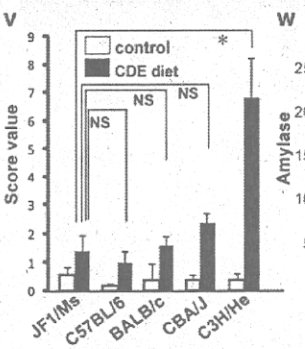


Figure 1 Histological changes, pathologic scores, and serum amylase levels in pancreas with and without cerulein treatment. (**a, b, e, f, i, j, m, n, q, r**) Saline-treated mice. (**c, d, g, h, k, l, o, p, s, t**) Cerulein-treated mice. (**b, d, f, h, j, l, n, p, r, t**) Higher magnification of areas indicated in (**a, c, e, g, i, k, m, o, q, s**). (**u**) Pathologic scores calculated based on five parameters: edema (E), hemorrhage (H), infiltrates (I), vacuolization (V), necrosis (N) of acinar cells. (**t**) Scores: total scores derived from the evaluation of five parameters. (**v**) Column graph of pathologic scores shown as mean ± s.d. (error bar). (**w**) Serum amylase levels. Black arrows indicate respective inflammatory changes. Scale bar: 50 μm. *$P<0.05$. NS: no significance.



u Pathologic scores in CDE-diet acute pancreatitis

M. Strain	Treat	No. of Mice	E	H	I	V	N	Total Scores	Mean ± SD
JF1/Ms	N. Diet	5	2	1	0	0	0	3	0.6 ± 0.24
	CDE	5	2	2	0	3	0	7	1.4 ± 0.54
C57BL/6	N. Diet	5	1	0	0	0	0	1	0.2 ± 0.07
	CDE	5	3	0	0	2	0	5	1.0 ± 0.38
BALB/c	N. Diet	5	1	0	0	1	0	2	0.4 ± 0.57
	CDE	5	2	6	0	0	0	8	1.6 ± 0.32
CBA/J	N. Diet	5	2	0	0	0	0	2	0.4 ± 0.18
	CDE	5	3	3	0	1	5	12	2.4 ± 0.42
C3H/He	N. Diet	5	2	0	0	0	0	2	0.4 ± 0.2
	CDE	5	7	6	2	0	19	34	6.8 ± 1.40



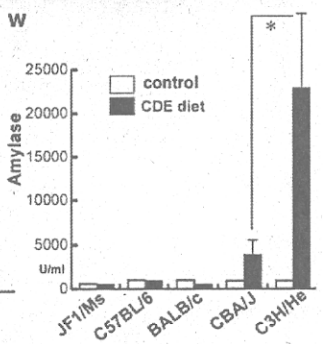


Figure 2 Histological changes, pathologic scores, and serum amylase levels in pancreas with and without CDE diet-induced acute pancreatitis. (**a, b, e, f, i, j, m, n, q, r**) Saline-treated mice. (**c, d, g, h, k, l, o, p, s, t**) Cerulein-treated mice. (**b, d, f, h, j, l, n, p, r, t**) Higher magnification of areas indicated in (**a, c, e, g, i, k, m, o, q, s**). (**u**) Pathologic scores calculated based on five parameters: edema (E), hemorrhage (H), infiltrates (I), vacuolization (V), necrosis (N) of acinar cells. (**v**) Column graph of the pathologic scores shown as mean ± s.d. (error bar). (**w**) Serum amylase levels. Black arrows indicate respective inflammatory changes. Scale bar: 50 μm. $^*P < 0.05$. NS: no significance.

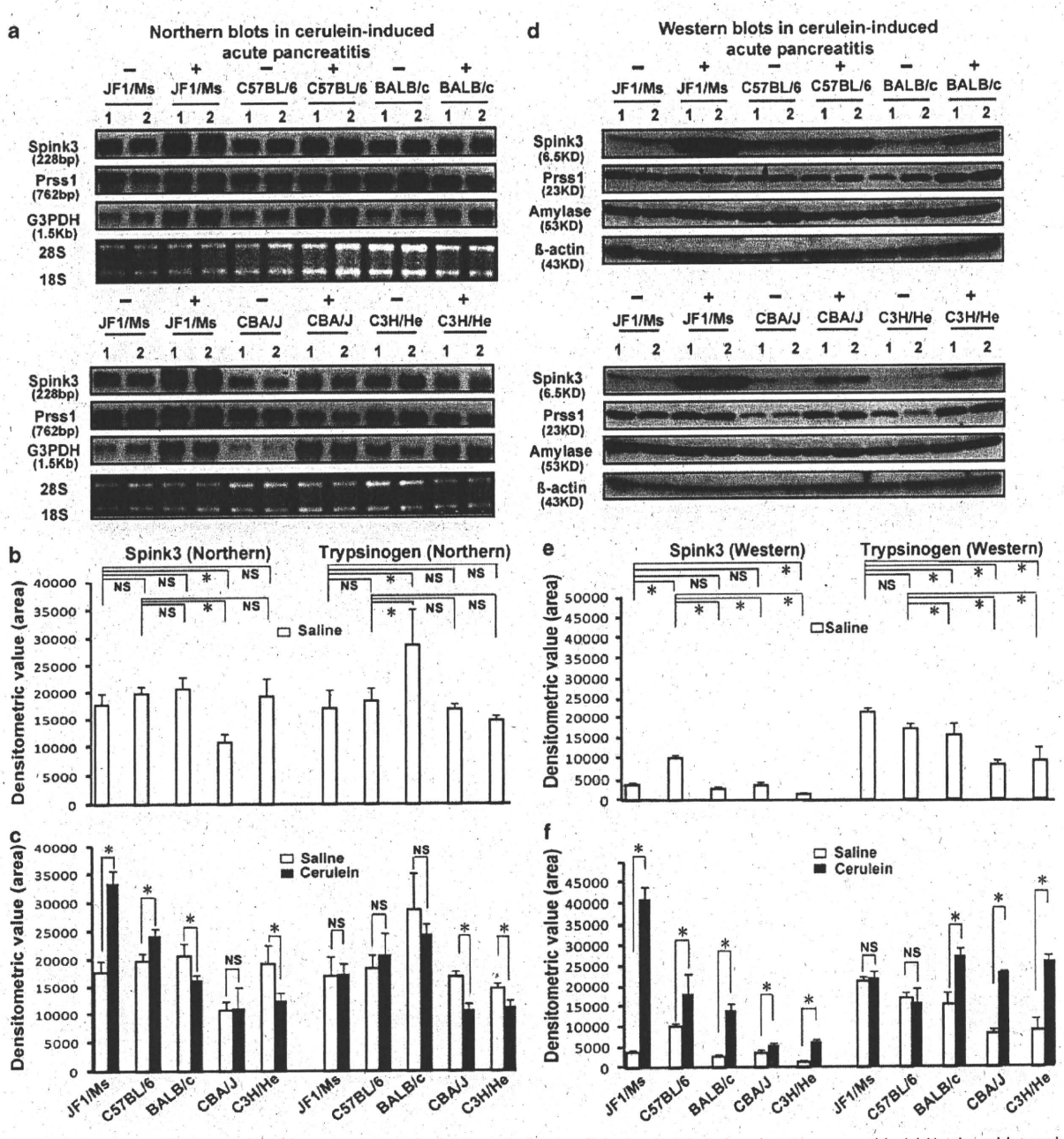


Figure 3 Northern and western blots analyses on Spink3, Prss1, and amylase expressions in cerulein-induced acute pancreatitis. (**a**) Northern blot analysis; 18S and 28S were used as loading controls because G3PDH expression was induced by cerulein treatment. The same JF1 samples were arranged in the first four lanes of northern blots. (**b**) Densitometric analysis for Spink3 and Prss1 expressions without cerulein induction in northern blot. (**c**) Densitometric analysis for Spink3 and Prss1 expressions with cerulein induction in northern blot. (**d**) Western blot analysis. β-actin was used as a loading control. The same JF1 samples were arranged in the first four lanes of western blots. (**e**) Densitometric analysis for Spink3 and Prss1 expressions after cerulein induction in western blot. (**f**) Densitometric analysis for Spink3 and Prss1 expressions with cerulein induction in western blot. Unfilled bars represent untreated strains of mice, whereas black bars indicate the treated strains. '−': saline treatment. '+': cerulein treatment. *$P<0.05$. NS: no significance.

Levels of Prss1 mRNA in JF1 and C57BL/6 strains were lower than those in other strains under a normal diet (Figure 5a and b). After CDE diet, levels of Prss1 mRNA were decreased in C57BL/6, BALB/c, CBA/J, and C3H/HeJ mice (Figure 4a and c). Protein expression for Spink3 and Prss1 was examined by western blot analyses. Under normal diet, the levels of Spink3 were again higher in JF1 and C57BL/6J than those in other three strains (Figure 4d and e). After CDE

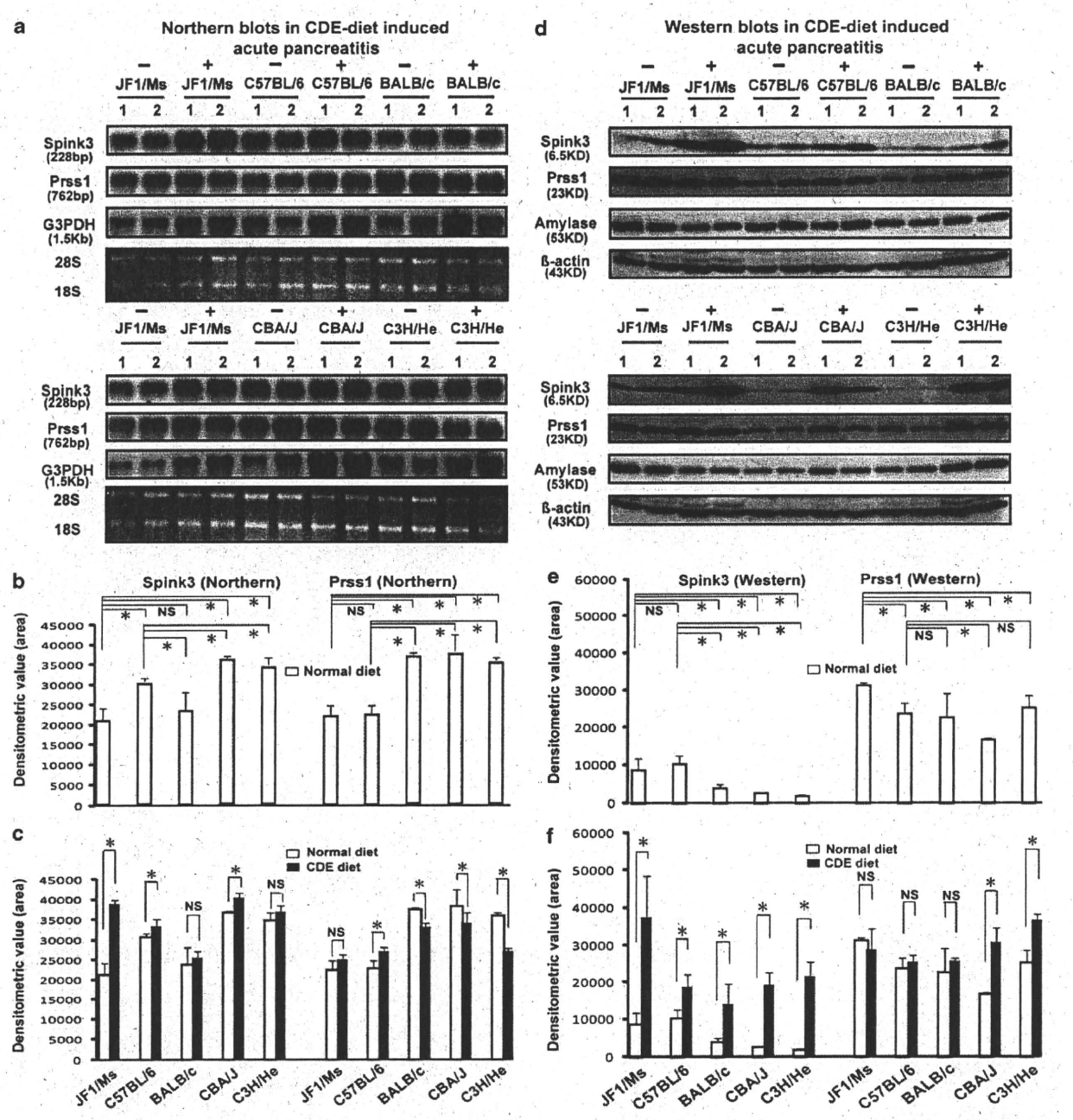


Figure 4 Northern and western blot analyses on Spink3, Prss1, and amylase expressions in CDE diet-induced acute pancreatitis. (**a**) Northern blot analysis; 18S and 28S were used as loading controls because G3PDH expression was induced by cerulein treatment. The same JF1 samples were arranged in the first four lanes of northern blots. (**b**) Densitometric analysis for Spink3 and Prss1 expressions without CDE diet in northern blot. (**c**) Densitometric analysis for Spink3 and Prss1 expressions with CDE diet in northern blot. (**d**) Western blot analysis. β-actin was used as a loading control. The same JF1 samples were arranged in the first four lanes of western blots. (**e**) Densitometric analysis for Spink3 and Prss1 expressions without CDE diet in western blot. (**f**) Densitometric analysis for Spink3 and Prss1 expressions with CDE diet in western blot. Unfilled bars represent untreated strains of mice, whereas black bars indicate the treated strains. $^*P<0.05$. NS: no significance.

diet, Spink3 expression increased significantly in JF1, but moderately in other strains (Figure 4d and f).

The level of Prss1 protein expression was lower in CBA/J than those in other four strains without CDE diet (Figure 4d and e). With CDE diet, Prss1 expression increased significantly in CBA/J and C3H/HeJ, whereas expression remained unchanged in JF1, C57BL/6J, and BALB/c mice (Figure 4d and f). These results also suggest that the induction levels of Spink3 and Prss1 are negatively and positively related to susceptibility of CDE-induced pancreatitis, respectively.

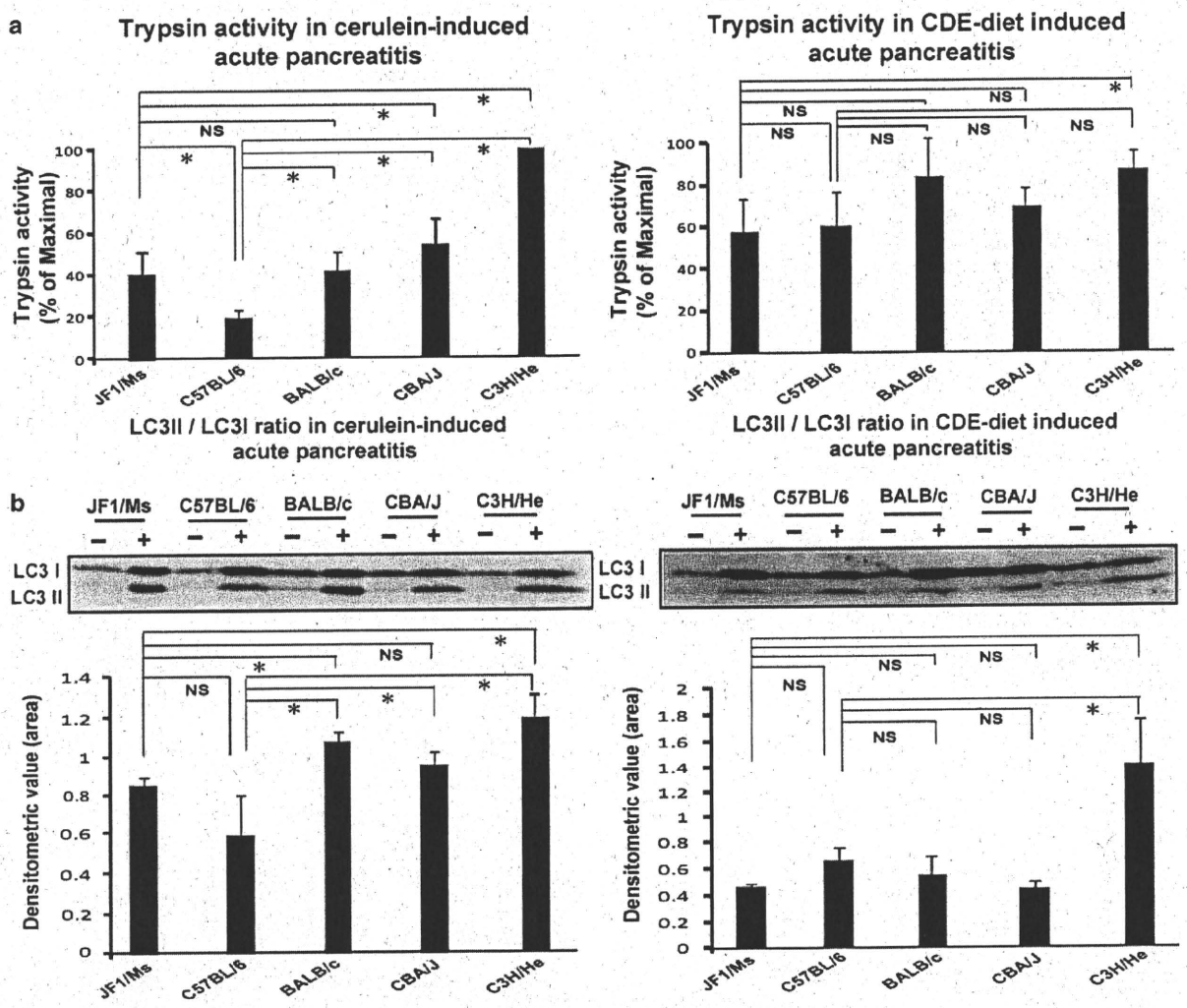


Figure 5 Trypsin activity and LC3-II expression. (**a**) Trypsin activity in cerulein-induced and CDE-diet-induced acute pancreatitis. Trypsin activity coincided with protein expression level of Prss1. (**b**) LC3-II/LC3-I ratio in cerulein-induced and CDE diet-induced acute pancreatitis. LC3-II levels were inversely related to Spink3 levels when treated with cerulein, suggesting the function of Spink3 as an inhibitor of autophagy.

Trypsin Activity

As activation or extent of trypsinogen to trypsin during initiation of acute pancreatitis is associated with the severity of acinar cell injury, we examined the trypsin activity level within pancreatic tissue homogenates among five mouse strains. The highest activity was shown in C3H/HeJ, followed by CBA/J, BALB/c, JF1, whereas C57BL/6J displayed the lowest value in cerulein-induced pacreatitis (Figure 5a, left panel). In CDE diet, trypsin activity level was significantly higher in C3H/HeJ than those in other strains (Figure 5a, right panel). Not surprisingly, the variation of trypsin activity was consistent with the expression pattern of trypsinogen among the five mouse strains.

Expression of LC3, an Autophagic Indicator

To approve that Spink3 functions also as a suppressor of autophagy, we checked in the five mouse strains the expression level of microtubule-associated protein 1 LC3. As LC3-I is converted into LC3-II during autophagosome formation, we determined the LC3-II/LC3-I ratio by densitometric analysis. As shown in Figure 5b, the ratio was higher in C3H/HeJ, BALB/c, and CBA/J than that in JF1 and C57BL/6J when treated with cerulein, showing that the mouse strain with higher Spink3 expression possessed low level of LC3-II, meaning less severe degree of autophagy.

Nucleotide Sequences of Prss1 and Spink3 cDNAs

To examine whether there was any nucleotide changes in coding regions of the *Prss1* and *Spink3* genes, we sequenced cDNA of these two genes isolated from all five strains. Nucleotide sequences of *Prss1* and *Spink3* cDNAs were exactly the same in four strains of mice: C57BL/6J, CBA/J, BALB/c, and C3H/HeJ. In contrast, in the 807 bp of the Prss1

Table 1 Single-nucleotide polymorphisms in JF1 cDNA *vs* B6

Prss1 (807 bp)			Spink3 (284 bp)		
Position	Nucleotide change	Amino-acid change	Position	Nucleotide change	Amino-acid change
Exon 1	15G>C	No	Exon 3	192G>A	No
Exon 3	342A>T	No	Exon 3	198C>T	No
Exon 4	516G>A	No	Exon 4	279A>G	No
Exon 4	519T>C	No			
Exon 4	568G>A	E190K			

JF1, Japanese Fancy Mouse 1. E190A, substitution of the glutamic acid (E) at amino-acid position 190 with lysine (K).

cDNA sequence of JF1 mice, five nucleotide changes were found (Table 1). However, only the alteration, G to A at position 568, resulted in substitution of glutamic acid with lysine at amino-acid position 190 (Table 1). On the other hand, three nucleotide changes were found within the 284 bp region of Spink3 cDNA of JF1 mice without an amino-acid substitution (Table 1).

Nucleotide Sequences of 3 kb Upstream Regions of Spink3 and Prss1 Genes

As expression patterns of Spink3 and Prss1 differed among mouse strains, we sequenced the approximately 3 kb promoter regions of Spink3 and Prss1 genes. We chose JF1 and C3H/HeJ as representatives of resistant and susceptible mouse strains, respectively. As the most conserved binding sites for transcription factors in pancreas-specific genes reside within the 1 kb upstream region, we focused on the 3 kb upstream regions of both Spink3 and Prss1 genes in this study. All of the sequence data were aligned and compared with the corresponding public database for C57BL/6J mice. For the upstream nucleotide sequence of the Spink3 gene, eight nucleotide changes (8/3000 = 0.27%) were found in C3H/HeJ *vs* C57BL/6J, whereas 92 nucleotide changes (92/3000 = 3.07%) appeared in JF1 *vs* C57BL/6J mice, representing a huge difference in the 3 kb upstream of the transcription start site in JF1 mice (Supplementary Figure 2). Notably, almost half of nucleotide changes[30] were found within the 1 kb upstream region in JF1 (Supplementary Figure 2). Several conserved motifs for transcription factors[31–36] were found in the 3 kb upstream regions of Spink3 genes as summarized in Supplementary Figure 2. Interestingly, an additional 10 bp was found between −372 and −381 in JF1 mice, although a conserved motif has not been reported around this region.

With respect to the Prss1 gene, 46 (46/3000 = 1.53%) or 39 (39/3000 = 1.30%) nucleotide changes were identified in C3H/He or JF1 strains against C57BL/6J mice, respectively (Supplementary Figure 3). However, the region between −3000 and −2300 bp of C57BL/6J was the same as that of JF1, but not of C3H. On the other hand, the region between −2300 and −1 bp of C57BL/6J mice was similar to that of C3H mice. Three binding sites were found in the 1 kb upstream region of the Prss1 gene[31,37] (Supplementary Figure 3). All these sequences are identical in all strains except one nucleotide change at −192 of the binding site (ATCACCTGCT) for nuclear protein in JF1 mice.

DISCUSSION

In this study, we showed strain differences using two models of experimental acute pancreatitis and identified a negative and positive relationship regarding the expression levels of Spink3 and Prss1, respectively, in the susceptibility to experimental acute pancreatitis. In addition, we showed that sequence differences in the promoter region of the Spink3 gene was significant between JF1 and other laboratory mouse strains, suggesting that differences in gene regulation are connected to a susceptibility to induced acute pancreatitis.

Pathologically, there were qualitative differences between cerulein- and CDE diet-induced pancreatitis in addition to the severity of pancreatitis. In the CDE diet model, much more hemorrhagic legions were noticed, as has been reported before.[28] This difference may be caused by different induction mechanisms in the early stages of acute pancreatitis. Cerulein is a cholecystokinin analog that can stimulate pancreatic acinar cells to secrete digestive enzymes. In supramaximal stimulation by cerulein, secretory activity increases dramatically, but membrane recruitment is insufficient for a strong demand of zymogen granule membrane, resulting in an inhibition of exocytosis at the luminal plasma membrane. On the other hand, a more likely target of ethionine is phospholipid metabolism of membranes that are involved in the processes of intracellular transport and secretion of pancreatic enzymes. Feeding a choline-deficient diet potentiated the activity of ethionine, because a choline-deficient diet also induced changes in membrane phospholipids of cellular organelles.[38–40] Although the detailed mechanism for hemorrhagic lesions remains unclear, destruction of the elastic tissue of the intrapancreatic vessels may also occur in such a situation.

In two experimental models, we observed similar strain differences in disease susceptibility, which were most severe in C3H/HeJ and CBA/J strains, moderate in BALB/c mice,

and mildest in C57BL/6J and JF1 strains. In human beings, a relationship between the PRSS1 gene mutations and the onset of pancreatitis has been established by many investigations.[21,30,41,42] Thus, it is possible that differences in primary structure or expression of the Prss1 gene are responsible for strain differences. Although we found one single-nucleotide polymorphism (SNP) in exon 4 that resulted in substitution of glutamic acid with lysine at amino-acid position 190 in JF1 mice, this mutation was not observed in the PRSS1 gene of human patients, suggesting that the coding region of the Prss1 gene is not related to the susceptibility to pancreatitis among these strains.

Interestingly, Prss1 protein expression was obviously increased in BALB/c, CBA/J, and C3H/HeJ mice, but not in JF1 and C57BL/6J mice with cerulein treatment. This high Prss1 expression coincided with a high trypsin activity. Many nucleotide changes found in the 3 kb upstream region of the Prss1 gene among C57BL/6J, C3H/HeJ, and JF1 strains may be responsible for different expression level.

Differences in the primary structure or expression of the Spink3 gene may also be involved in susceptibility to the development of pancreatitis. However, we could not find any SNPs with an amino-acid substitution between JF1 and other strains of mice. Thus, the coding region of the Spink3 gene was not related to pancreatitis susceptibility. Meaningfully, we found a significantly higher expression of Spink3 in JF1 and C57BL/6J mice under normal feeding (Figure 3a and b). Furthermore, Spink3 expression was strongly augmented in JF1 mice with cerulein treatment. This high Spink3 expression was associated with low level of LC3-II expression, implying the function of Spink3 as an inhibitor of autophagy. As shown in Supplementary Figure 2, we found significant nucleotide changes in the 3 kb upstream region of the Spink3 gene of JF1 mice. It is of interest that an additional 10 bp was inserted between −372 and −381 region in JF1 mice, although a conserved motif has not been reported around this region. Therefore, this region might be involved in the regulation of gene expression under cerulein stimulation. Taking these into account, the mechanisms by which Prss1 and Spink3 might be influencing the susceptibility are proposed here in a molecular model shown in Figure 6. In resistant strains, increased Spink3 expression caused by cerulein or CDE diet suppresses autophagy, leading to reduced activation of trypsinogen. In addition, Spink3 can bind to trypsin to inhibit its activity directly. Thus, both enhanced Spink3 and low Prss1 expression result in reduced trypsin activity, leading to less severe acute pancreatitis. In susceptible strains, low level of Spink3 expression results in enhanced autophagy, causing the conversion of trypsinogen to trypsin, and in low-level inhibition of trypsin. Thus, both high Prss1 and low Spink3 expressions result in increased trypsin activity, leading to more severe acute pancreatitis.

After activation of trypsinogen, inflammation is eventually induced in both models. Therefore, different responses in inflammatory factors, such as NF-κB, TNF-α, IL-1,

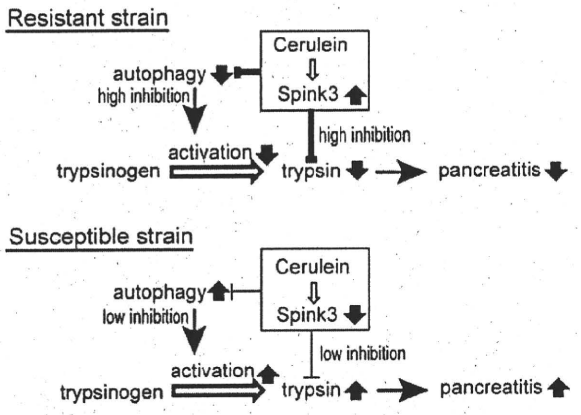


Figure 6 Proposed model for the function of Prss1 and/or Spink3 on susceptibility of acute pancreatitis. In resistant strains, increased Spink3 expression caused by cerulein suppresses autophagy, and trypsin activity, leading to less severe acute pancreatitis. In susceptible strains, low expression of Spink3 and high expression of Prss1 result in enhanced autophagy, causing the conversion of trypsinogen to trypsin and in low-level inhibition of trypsin, thus leading to more severe acute pancreatitis.

Heat Shock Protein, NO, or TLR4, among strains may be involved in pancreatitis susceptibility. Actually, inflammatory responses are different from one strain to another and a given strain can have variable inflammatory responses from one form of damage to another. For example, inbred mice varied significantly in their susceptibility to cigarette smoke-induced emphysema.[43,44] Further studies will be required to analyze genetic mechanisms for differences in inflammatory responses.

Advances in molecular physiology indicate the mouse to be an ideal investigative model to determine genomic variants that affect susceptibility to disease. This model offers the advantage of studying a large number of genetically identical animals under controlled conditions. Animal studies complement human studies by introducing an experimental control and the opportunity to pursue functional genomics and expression studies at the level of organs, tissues, or cells. Molecular mechanisms for disease resistance/susceptibility may differ among strains, and the JF1 strain may give a unique opportunity to examine such mechanisms that may not be found in other laboratory mouse strains.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Establishment of germline-competent embryonic stem cell lines from the MSM/Ms strain

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Abstract MSM/Ms is an inbred mouse strain established from the Japanese wild mouse, *Mus musculus molossinus*, which has been phylogenetically distinct from common laboratory mouse strains for about 1 million years. The nucleotide substitution rate between MSM/Ms and C57BL/6 is estimated to be 0.96%. MSM/Ms mice display unique characteristics not observed in the commonly used laboratory strains, including an extremely low incidence of tumor development, high locomotor activity, and resistance to high-fat-diet-induced diabetes. Thus, functional genomic analyses using MSM/Ms should provide a powerful tool for the identification of novel phenotypes and gene functions. We report here the derivation of germline-competent embryonic stem (ES) cell lines from MSM/Ms blastocysts, allowing genetic manipulation of the *M. m. molossinus* genome. Fifteen blastocysts were cultured in ES cell medium and three ES lines, Mol/MSM-1, -2, and -3, were established. They were tested for germline competency by aggregation with ICR morulae and germline chimeras were obtained from all three lines. We also injected Mol/MSM-1 ES cells into blastocysts of ICR or C57BL/6 × BDF1 mice and found that blastocyst injection resulted in a higher production rate of chimeric mice than did aggregation. Furthermore, Mol/MSM-1 subclones electroporated with a gene trap vector were also highly efficient at producing germline chimeras using C57BL/6 × BDF1 blastocyst injection. This Mol/MSM-1 ES line should provide an excellent new tool allowing the genetic manipulation of the MSM/Ms genome.

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Introduction

Mus musculus is divided into at least four major subspecies: *M. m. domesticus*, *M. m. bactrianus*, *M. m. musculus*, and *M. m. castaneus* (Bonhomme and Guénet 1996; Moriwaki et al. 1994). An additional subspecies, known as *M. m. molossinus,* is found in Japan. This is not an independent subspecies but arose through hybridization between *M. m. musculus* and *M. m. castaneus* (Yonekawa et al. 1988). MSM/Ms is a mouse strain derived from Japanese *M. m. molossinus* wild mice collected in 1978 in Mishima, Japan (Moriwaki et al. 1994). This strain has been inbred for 100 generations and can therefore be regarded as an inbred strain of *M. m. molossinus*.

The MSM/Ms strain is widely used for linkage analysis and positional cloning because of its genetic divergence from common laboratory mouse strains that are derived predominantly from *M. m. domesticus*. Recently, an arrayed bacterial artificial chromosome (BAC) library of the MSM/Ms genome has been constructed, and BAC clone-end sequence analysis revealed that 0.96% of the nucleotides in the MSM/Ms genome differed from those in the common laboratory mouse